Three-dimensional reconstruction and morphologic measurements of human embryonic hearts: a new diagnostic and quantitative method applicable to fetuses younger than 13 weeks of gestation.

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Three-dimensional reconstruction and morphological measurements of human embryonic hearts. A new diagnostic and quantitative method applicable to fetuses younger than 11 weeks of gestational age

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Abstract

Improvements in the diagnosis of congenital malformations explain the increasing early termination of pregnancies. Before 13 weeks of gestation, an accurate *in vivo* anatomical diagnosis cannot currently be made in all fetuses with the imaging instrumentation available. Anatomo-pathological examinations remain the gold standard to make accurate diagnoses, although they reach limits between 9 and 13 weeks of gestation. We present the first results of a methodology that can be applied routinely, using standard histological section, enabling the reconstruction, visual estimate and quantitative analysis of 13 weeks of age human embryonic cardiac structures. The cardiac blocks were fixed, included in paraffin and entirely sliced by a microtome. One slice out of 10 was topographically colored and digitized on an optical microscope. The cardiac volume was recovered by a semi-automatic realignment of the sections. Another semi-automatic procedure allowed extracting and labeling of the cardiac structures from the volume. The structures were studied with display tools, disclosing the internal and external cardiac components, and enabling the determination of size, thickness and precise position of the ventricles, atria and large vessels. This pilot study confirmed that a new 3-D reconstruction and visualization method enabled to make accurate diagnoses, including in embryos <13 weeks old. Its implementation at earlier stages of embryogenesis will provide a clearer view of cardiac development.

Keys words: congenital heart disease; fetal malformation; cardiac imaging; embryonic heart; 3-D reconstruction; 3-D visualization

Introduction

The prevalence of congenital heart defects, the most common congenital anomaly observed in newborns, has been reported to be 5-10 per 1000 births. [1, 2] The prevalence of congenital heart defects identified in aborted fetuses or in fetuses who have died *in utero* was estimated to be five-fold higher than in live infants. [1, 2] Heart defects are responsible for 20% of the neonatal deaths due to congenital anomalies. [3]

The complexity of the cardiac anatomy and the wide variety of cardiac defects mandate an accurate anatomical diagnosis, with a view to confirm the tentative echographic diagnosis that had prompted the termination of pregnancy. Verification of the cardiac anatomy is also needed when an extracardiac anomaly has been detected, as the secondary finding of an associated cardiac defect may reorient the initial diagnosis. Several large studies performed in fetus with congenital heart anomalies, aborted either deliberately or spontaneously, have compared the prenatal diagnosis with the *post mortem* findings. [4-7] At average gestational ages as late as 20 to 23 weeks, the main prenatal diagnosis was revised by the autopsy in 20-37% of cases, particularly in syndromes associated with multiple malformations.

As a result of advances in diagnostic methods, we have observed a steady decline in the gestational age at which fetuses with congenital anomalies are aborted. Major structural anomalies are now detected in the prenatal period, as early as 11 weeks of age (WOA), representing medical indications for the termination of pregnancy during early fetal development. Since, at this early gestational age, ultrasound examinations lack the critical accuracy, needed in the diagnosis of complex congenital cardiac malformations, has become particularly important to detect anomalies in small fetuses following pregnancies of short duration. However, with the imaging instrumentation currently available, an accurate anatomical cardiac diagnosis cannot be made *in vivo* in fetuses before 13 weeks of gestation.

Ex vivo or *in vitro* imaging techniques have not been adapted to the dimensions of the organs under examination. Therefore, the heart is too small for reliable examinations by echocardiographic, [8, 9] or micro-magnetic resonance imaging (MRI), [10-12] or by micro-computer tomography**.** [13] On the other hand, it is too large to be studied by confocal microscopy. [14-16]

Ultimately, pathological examination remains the gold standard allowing the making of accurate diagnoses. However, this method reaches its own limits between 9 and 13 weeks of gestation, when the embryo is between 25 and 70 mm in length. At this point, the examination of isolated organs is a challenge, particularly of the heart, which measures between 3 and 8 mm.

The aim of this report is to describe the tools and analytical method, which can be applied routinely, using standard histologic section, enabling the reconstruction, visual estimate and quantitative analysis of the human embryonic cardiac structures. This is the first description of 3-dimensional (3-D) reconstruction allowing a diagnostic analysis of fetal human hearts ≤ 13 WOA.

Methods

Anatomo-pathological background

This study was reviewed and approved by the Bioethics Medical Committee of our university hospital. Abortion specimens stored in 4% para-formaldehyde, usually consisting of embryo and placenta, were routinely examined together or separately in our department of pathology. A whole body photograph was obtained before the beginning of any dissection. The length of the embryo was measured, and a detailed evaluation of the facial characteristics, limbs and body features was performed under a Wild M7A zoom dissecting stereo-microscope. Body

weight, crown-rump and foot lengths were recorded and compared with standard charts. External features were detailed using a magnifying lens.

A limited autopsy was performed on fetuses ≤ 13 weeks of gestation. The thoracic and abdominal cavities were examined via a straight midline incision and a transverse incision just above the level umbilicus. After removal of the chest wall, the internal organs were examined with a dissecting microscope with a camera attachment (Wild MPS45 Photoautomat and Polaroïd CB 101 back) allowing documentation with color photographs. Location of the viscera was noted before the undertaking of meticulous dissection, using autopsy techniques identical to those used in the perinatal period, for identification of all major malformations. The thoracic organs were removed *en bloc*, and the heart was separated from the respiratory tract. Photographs of the external surfaces of the heart were obtained for documentation and further reference, and heart weight and size were measured. The heart was stored in 4% paraformaldehyde for several days.

The specimen used as an example in this article was the heart of human fetus, 68 mm in crown/rump length, corresponding to 74 days post ovulation, or 11 weeks of gestation, prepared as described earlier. This fetus's external appearance was normal. Its dissection revealed no malformation. The heart weighed 0.2 g, and measured 9 mm x 8 mm (figure 1). The whole heart was dehydrated with ethanol and embedded in paraffin according to standard procedures. Serial 10 µm-thick transversal sections were performed with a Leica RM 2145 microtome (Meyer Instruments, Inc, Houston, TX). One out of 10 sections was stained in a standard fashion with hematoxylin-eosin. The colored sections were digitized on an IKAROS 3 V 4.33 optical microscope (Metasystems, Inc., Broadview Heights, OH) (figure 2). During digitization, we performed a first visual alignment of the sections since the sections were placed on the slides without attention to their initial orientation. The inter-section spacing was known by the acquisition protocol. However, the resolution within a section was estimated by

digitizing a scale graduated in mm on one of the images during the acquisition process. This calibration process yielded a 22.3 µm pixel size within the section and a 100 µm inter-section distance.

3-D image processing

The cardiac volume was reconstructed from the acquired sections in 3 main steps, as described by Baldock et al.: [17] 1) realignment of the histologic sections, 2) extraction and annotation of the useful information, and 3) volume recovering. The 3 steps require several techniques detailed in the following paragraphs. The image processing procedures used for the reconstruction are standard and easy to develop. Such procedures could readily be integrated as plug-ins into standard image processing software packages like NIH image (or ImageJ in PC applications) or equivalent software, which is accessible at <http://rsb.info.nih.gov/>.

Realignment of the histologic section

Due the acquisition methodology, the heart sections do not maintain a fixed position over several layers. The reconstruction of the heart morphology in 3 dimensions requires a precise superposed realignment of the sections. This realignment represents 3 major challenges: 1) since there is no external reference point between sections, the alignment method has to use the intrinsic information contained within each section; 2) because of subtle changes in cardiac morphology from one section to the next, the alignment method cannot rely on a precise similarity among sections; 3) the microtome may cause local distortions of the sections, which are not predictably at the same location in each section.

Based on a review of several alignment methods, [18] we have implemented a rigid matching technique to eliminate further distortions of the section contents. However, because of the non-similarities between sections, we have developed a semi-automatic two-steps matching technique:

1) A first automatic rigid alignment method applied to the denser structures, e.g. the main arteries and ventricles, which are little or not at all distorted by the microtome. These structures are characterized by deep gray level values on the section, and can, therefore, be extracted easily by a simple threshold value. A classical method based on the geometrical moment computed on the extracted structures is applied directly to the images, without any point or contour extraction. [19] The examiner interaction is limited to the choice of threshold.

2) Persistent misalignments due to distortions or non-similarities between sections are manually corrected by an interactive, custom-developed, rigid fitting computer software. This software superimposes 2 consecutive sections and displays the differences between them. The examiner then corrects the misalignment by manually adjusting the discrepancies visually identified between images. This correction is then extrapolated to the other sections of the set (figure 3).

Extraction and annotation of the useful information

The goal of this procedural step is to isolate the ventricles, the atria, the aorta and the pulmonary artery. Due to the complex shapes and structures of the images (no distinct differences in values between the structures, fusion between structures, deformation of the thin atria wall, blood clots, etc.), no fully automatic extraction process and labeling procedure seemed adapted to the pursuit of our goal. As described previously, we have developed a semi-automatic segmentation and labeling technique:

First, the 3-D structures, including the ventricles and the main arteries, are directly extracted by a region-based technique. [20] The user chooses interactively and visually 2 threshold values including the ventricles and the main arteries. The image processing then isolates automatically and in 3-D the volume of the objects of interest. Second, a refinement of the threshold values allows the separation of the ventricles from the great arteries by the

same techniques. Third, the great arteries are separated section-by-section interactively. Finally, since extraction of the atria is complicated by their thin walls, complex shape and distortion during sectioning, we enhanced and manually delineated the overall atrial volume, section-by-section, using the Adobe Photoshop™ software (Adobe Systems Inc. San Jose, CA). Ultimately, the labeled cardiac structures are 1) atria, 2) ventricles, 3) aorta, and 4) pulmonary artery (figure 4).

Volume recovery

The alignment allows reconstruction of the volume by stacking the sections. Since this volume is not isotropic (pixel size 22.3 µm and inter-section distance 100 µm), resampling along the stack orientation was performed by interpolation on both the gray scale and on the labeled sections.

Results

Visual estimation of volumes

The cardiac volume was estimated visually as well as measured. The shape and topology of the cardiac structures can be visualized by 3-D methods. The internal cardiac structures can be also explored accurately by 2-D reslicing in any 3-D orientations.

3-D visualization

3-D voxel volumes are well adapted to an analysis by 3-D visualization methods. We used an improved volume-rendering Ray-Casting-based method, which allows the direct visualization of the surfaces within the voxel volume, without prior surface extraction. [21] This technique has been adapted to use simultaneously the information contained in the grayscale and the labeled volumes. This enables to visualize and estimate the size, thickness and relative positions of the various cardiac structures, both externally and internally (figures 5 & 6). This mode of presentation is particularly informative as it allows the visualization, by

transparency, of the position of the cardiac structures with respect to each other, for example the precise position of the aortic root and/or pulmonary artery with respect to the ventricles. This presentation merges directly with the descriptive principles of cardiac malformations in the form of atrio-ventricular or ventriculo-arterial concordance. The presence and magnitude of a ventriculo-arterial mismatch are identifiable and quantifiable, facilitating the distinction between Tetralogy of Fallot and double outlet right ventricle. The joint analysis of the internal anatomy allows an exact definition of the cardiac position and precise identification of the ventricles, further enhancing the diagnostic accuracy of the malformation. The precise identification of the atria is limited by the absence of common descriptive anatomic elements, such as appendage and pectinate muscles, within the reconstructed volume.

2-D reslicing

2-D reslicing means exploration of the reconstructed volume by interactively defined cut planes. The usual solution is to explore the volume in 3 perpendicular, user-defined sections, allowing the specification of morphology and spatial relationship between anatomical structures, for example between valves and papillary muscles. The user can select any component of the volume in the frontal or sagittal plane. For example, the morphology and relationship between sigmoid valves or between atrio-ventricular valves can be analyzed (figure 7-8). The user can also select any other plane in order to examine a specific structure in the optimal orientation. For example, the origin of the coronary arteries (figure 9), or a thin structure such as the interatrial septum (figure 10) can be isolated and analyzed accurately in the same plane.

This analytical method is of particular interest after the user has identified the type of cardiac malformation, since it enables the study of precise elements of cardiac anatomy, including integrity of the interventricular septum, origin of the coronary arteries, the attachment of cordae tendinae, etc. Furthermore, in contrast to standard methods of

pathological examination, an *a posteriori* reanalysis in other orientations, after an initial diagnosis has been made, remains possible. For example, when a diagnosis of Tetralogy of Fallot has been made, one can specifically reexamine the origin and course of the coronary arteries.

Anatomical measurements

The anatomical measurements are typically performed on the section, and limited to the dissection plane. Though surfaces and volumes can be measured, they require complicated stereological methods. With our method, the volume was reconstructed and calibrated, enabling repetitive measurements on the same structure, in any direction. For example, interventricular septal thickness can be measured in several selected planes (figure 11), in particular those used in fetal echocardiography. The reconstructed thickness varies between 410 and 540 μm in figure 11-A, and measures 535 μm in figure 11-B. Likewise, in a projection similar to that shown in figure 10, we have measured the aortic internal (500 μm) and external (1200 μm) diameters, and those of the pulmonary artery (550 μm and 1150 μm, respectively). These aortic diameters measurements are particularly important when left heart malformations are suspected. Furthermore, the measurements of surface and volume are immediately available. For example, the volumes of the ventricles and atria of the 11 WOA heart were approximately 28.5 mm^3 and 40.2 mm^3, respectively. This relative disproportion of the atrial dimension may be surprising compared to the adult heart, though supports some hypotheses of cardiac embryology.

Discussion

A certain number of pregnancies are terminated very early, because major morphologic (particularly of the brain and neural tube) or chromosomal anomalies have been detected. [22, 23] Cardiac, gastro-intestinal, renal or skeletal anomalies that may be associated are poorly

visible during the 1st trimester by traditional prenatal imaging methods. [6, 7, 24] In the study by Kaiser, out of 8 cardiac anomalies confirmed at autopsy, only one had been detected during gestation. [24] A *post mortem* pathological exploration remains an indispensable cross-examination after termination of pregnancy, to confirm and refine the tentative prenatal diagnosis. [4, 22, 24, 25] It is a standardized method, available in all hospitals, with high imaging resolution and appropriate for the fetal heart size. However, at very early developmental stages, the standard pathological methods are limited by the small size of the samples under analysis. [4] Furthermore, it is a destructive method, which limits the analysis to the selected sections. When planning the sections, the anatomo-pathologist is inevitably influenced by the prior tentative diagnosis, and makes a choice accordingly, which, if misguided, cannot be modified.

Alternate imaging methods

Most of the alternate imaging methods currently available, including photonic confocal microscopy [14-16, 26], ultrasound backscatter microscopy [8, 9] and optical coherence tomography [27-29] are not adapted to the size of the examination samples.

High-field micro-MRI has enabled the 3-D reconstruction of the heart and outflow tracts in fixed mouse embryos. [10] This technique, which does not damage or distort the specimen, allows a qualitative and quantitative 3-D analysis of the object. However, it requires highly specialized and dedicated equipment. In addition, a poor spatial resolution, low signal/noise ratio, and prolonged acquisition time do not produce images of sufficient quality to make accurate diagnoses of embryonic heart anomalies. [10-12]

We have made an attempt to apply micro-MRI in a heart other than the one described in this article, at the same stage (13 weeks) of gestational development. Two separate sets of 3-D images were acquired (figures 12-B & 12-C) with RMN Bruker Avance DRX 500, 7 Tesla instrumentation (Bruker BioSpin GmbH, Germany). A comparison of the images acquired by

micro-MRI with the sagittal image acquired with our method (figure 12-A) clearly shows the lower resolution and highly noisy images produced by the former, rendering the anatomical and topological analysis of the cardiac structures difficult. The conclusion drawn from this review of available imaging tools is that current anatomic studies continue to depend on histological sections, because of the resolution they provide. However, improvements are soon expected by the addition of a 3rd dimension by 3-D reconstruction.

The 3-D reconstruction of embryonic hearts has been attempted for over 120 years using wax, plastics, cardboard and, more recently, manual and virtual graphic techniques. [30-33] This 3-D reconstruction, by enhancing the perception, identification and interpretation of various structures, facilitates the understanding of cardiac malformations.

In 1984, Wenink and Chon showed that graphic reconstructions from embryonic porcine hearts were very similar to those obtained from electron microscopic preparations, though added complementary information. [34]

The actual techniques described in the literature differ mostly in the answer they offer to the key elements of the reconstructive process, including extraction and annotation of pertinent information, volume reconstruction, and visualization of the different structures.

With respect to extraction and annotation, the majority of investigators have used section-by-section manual techniques. [35-37] Though relatively simple, these techniques are quite labor-intensive when applied to large numbers of sections. Their automation is limited by difficulties in the outlining and labeling of complex and weakly contrasted structures. [14, 38] In contrast, our method utilizes semi-automatic extraction and labeling, which facilitates and accelerates the process significantly. Though the extraction of the atria remains entirely manual, its automation is currently being developed.

The reconstruction of volumes by alignment and superimposition of successive sections is technically challenging because, on the one hand, the sections created by the

microtome are haphazardly oriented and, on the other hand, the procedures of preparation and slicing are the source of distortions. [39, 40] Three main solutions have been developed: 1) an episcopic alignment of the images, which liberates from distortions and realignment difficulties. [35, 38] However, this requires special instrumentation and is limited by difficulties in obtaining a balanced staining and homogeneous impregnation of the specimen. [17] 2) the use of external markers included in the paraffin to assist in the realignment. [41] 3) alignment of the sections based strictly on the information contained in the images. The difficulty of this type of correction stems from the challenge in distinguishing between-sections changes due to the actual shape, versus distortions of the specimen. Most investigators realign the sections by highly time-consuming manual procedures. To our knowledge, a single team has proposed an automatic pre-alignment, followed by manual correction. [14] In our approach, which emulates that latter methodology, only a minor correction is needed after the initial automatic step.

The instruments of graphic visualization are highly dependent on the type of information available and extracted. Studies that have extracted the contours of the structures under examination, which are a majority, were only able to visualize the 3-D surfaces with the assistance of polygone imaging software. [14, 35-38] While this type of visualization faithfully recognizes the surface of the structures, the remainder of the information, including fine surface or internal details, or thickness and nature of the walls, is forever lost. Only we and others, [14, 38] who have reconstructed the entire volume, benefit from several modes of exploration of the external surfaces, as well as of the sections across their volume to visualize the internal details. The 3-D technique which we are proposing allows the visualization of the pericardium and endocardial surface by transparency, as well as a direct exploration of the volume by the creation of sections in any plane, or of perpendicular planes. This novelty represents an important methodological and diagnostic progress for the pathologist. The hope

of being able to section the specimen as many times as needed in all spatial dimensions and to finally reexamine it in its entirety has finally been fulfilled.

Study limitation

Although we recognize the preliminary nature of this pilot study, which was limited to the reconstruction of a single normal heart, our primary objective was to demonstrate the applicability of our method. The quality of the images we have obtained thus far leaves little doubt that the method will be applicable to the study of abnormal hearts.

Conclusions

Our work should be viewed as a pilot study of the feasibility of reconstructing cardiac structures from histologic sections of human embryos <13 WOA. Our purpose was to demonstrate that this method can be applied to routine clinical practice. Our technique has several advantages. The image reconstruction from histologic sections offers a higher resolution than other imaging techniques. The cardiac volume is reconstructed in its entirety while retaining all the information, and the volume can be explored and quantified with 2-D and 3-D imaging. Certain procedures need refinements in order to limit the human-computer interactions. Problems inherent to the acquisition of histologic sections, such as the distortion of thin atrial walls, have only been partially solved by the techniques presented in this article. Several solutions may be considered, such as improvements in the image processing by integration and modeling of the distortions, fundamental improvements in other imaging techniques, including ultrasound or micro-MRI, and/or the application of techniques of fusion of data collected by these various imaging methods. For example, non-distorted sections obtained by micro-MRI could be integrated with higher-resolution histologic sections.

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Figure legends

Figure 1: **External aspect of the heart.**

The scale at the bottom of the image is graduated in mm.

Figure 2: **Two sections of the 13-WOA embryonic heart.**

Left: the ventricles are surrounded by a portion of atrial wall.

Right: the section is through the atria, the aorta and the pulmonary artery. The pulmonary and aortic valves are visible in the center of the vessels. Several blood clots are present in the atria.

Figure 3: Outcome of the section realignment.

Left: two consecutive sections in their original position.

Right: the two sections have been aligned.

Figure 4: Segmentation and labeling of the cardiac structures.

A) Atria; B) ventricles; C) Aorta; D) pulmonary artery.

Figure 5: 3-D reconstruction of the volume. The endocardial surfaces are visible by transparency.

Figure 6: Oblique section through the ventricles showing the ventricular walls thickness. The endocardial surface and the pericardium of each ventricle are clearly distinguishable.

Figure 7: 3-D volume and reconstruction of a frontal view.

Figure 8: Perpendicular sections through the volume.

A: Initial view. **B** and **C**: reconstructed frontal and sagittal planes. Note the perpendicular view of the aortic semi lunar valves on the top left section.

Figure 9: Origin of both coronary arteries

Figure 10: Inter-atrial septum

Figure 11: Measurements of interventricular septal thickness in axial (A) and frontal (B) sections.

Figure 12: Comparisons among images obtained by our method (A), versus micro-MRI at a 256 x 256 slice resolution, 156 µm slice thickness, and 97.6 µm pixel size (B), versus micro-MRI at a 512 x 512 slice resolution, 48.8 µm slice thickness, and 48.8 µm pixel size (C).

Figure 1

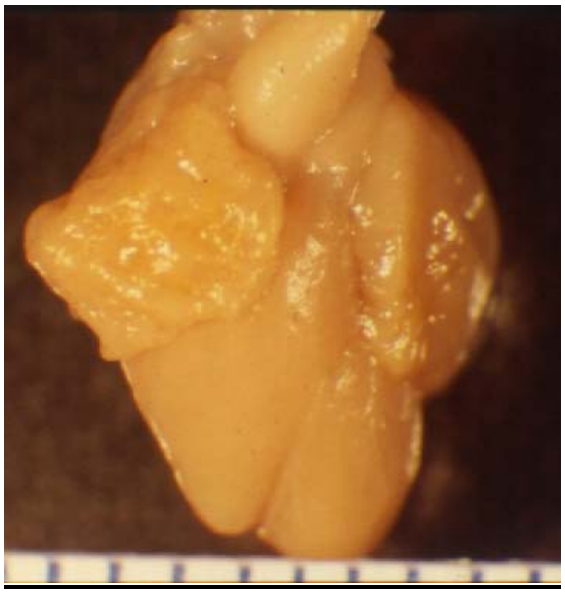

Figure 2

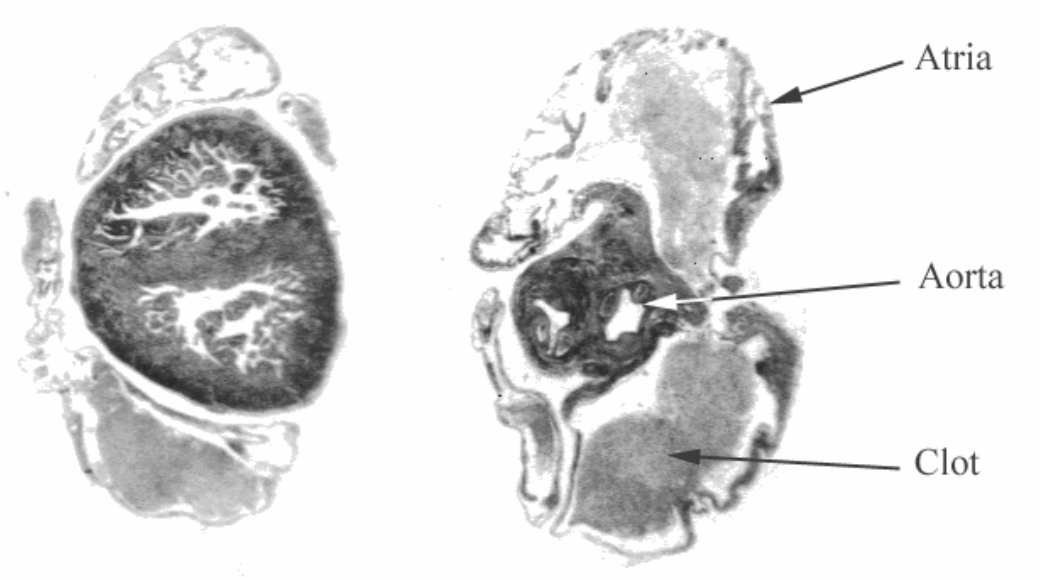

Figure 3

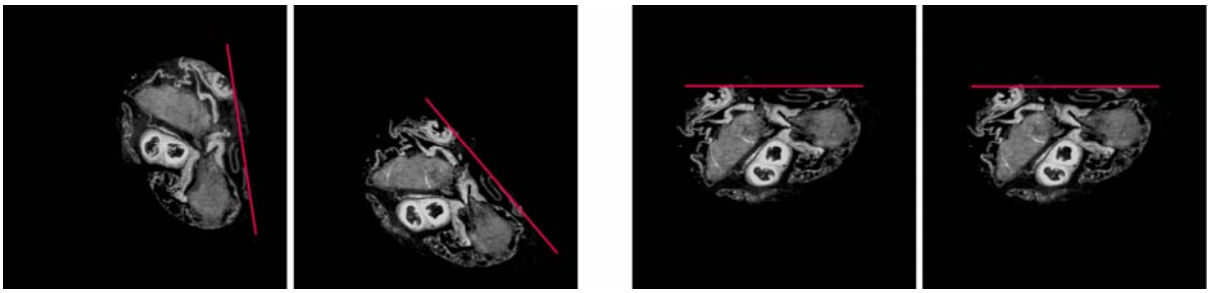

Figure 4

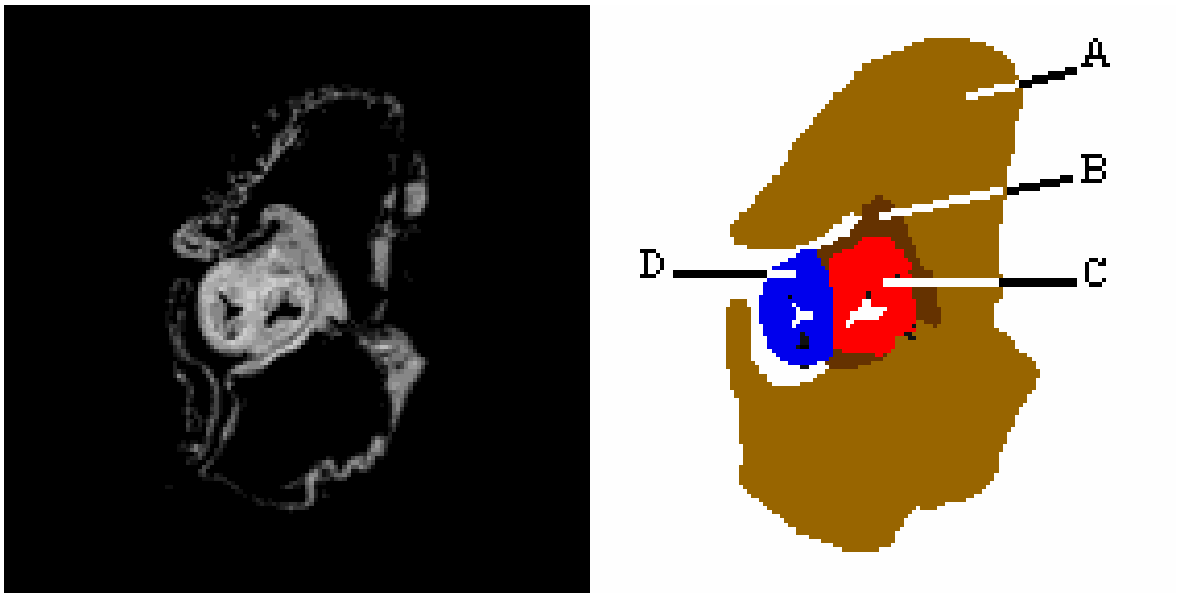

Figure 5

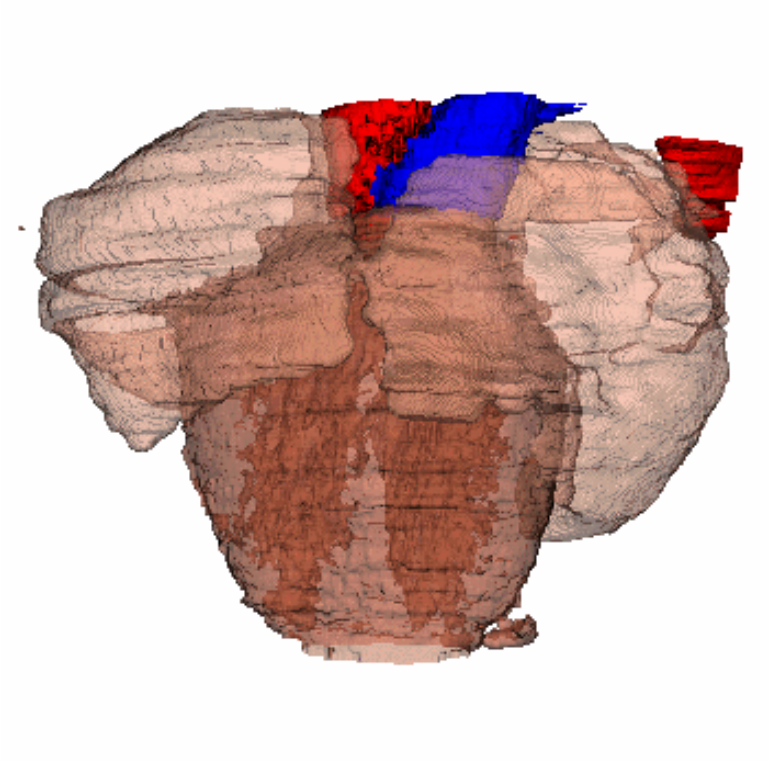

Figure 6

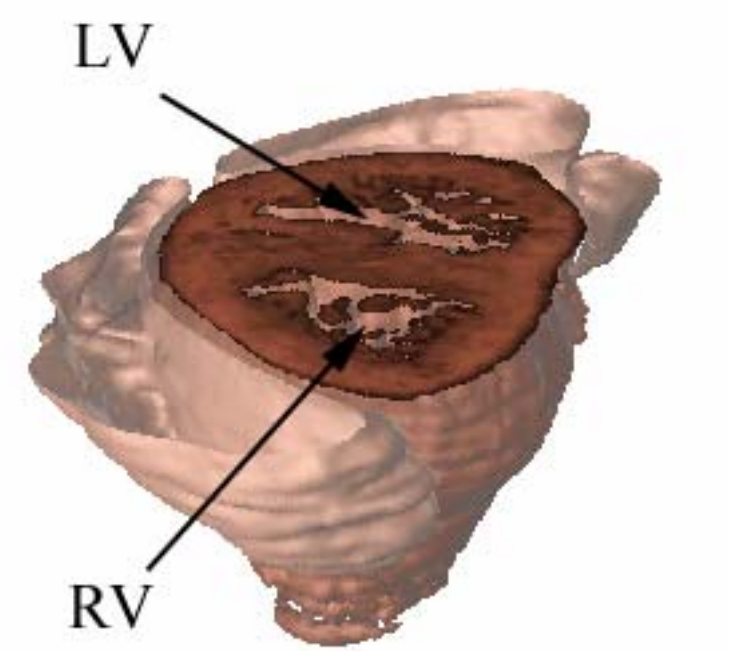

Figure 7

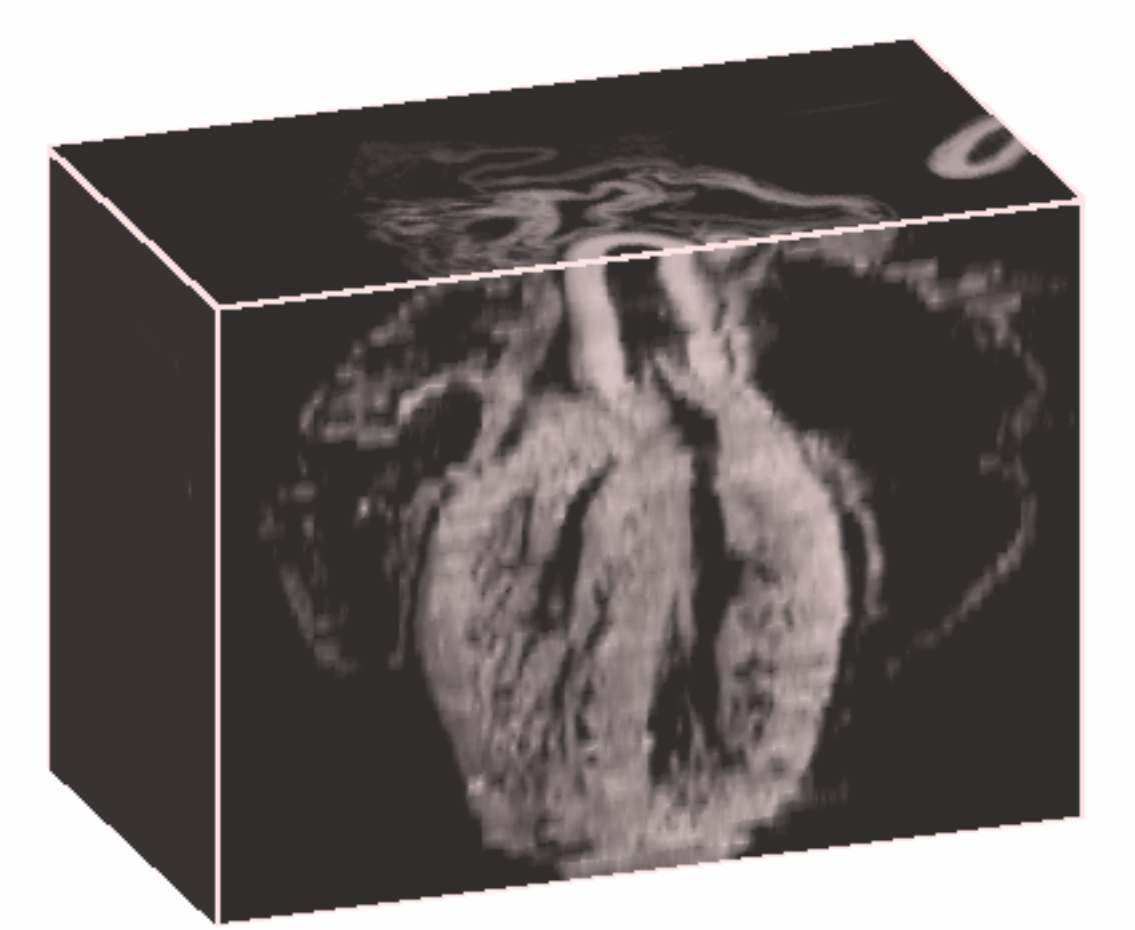

Figure 8

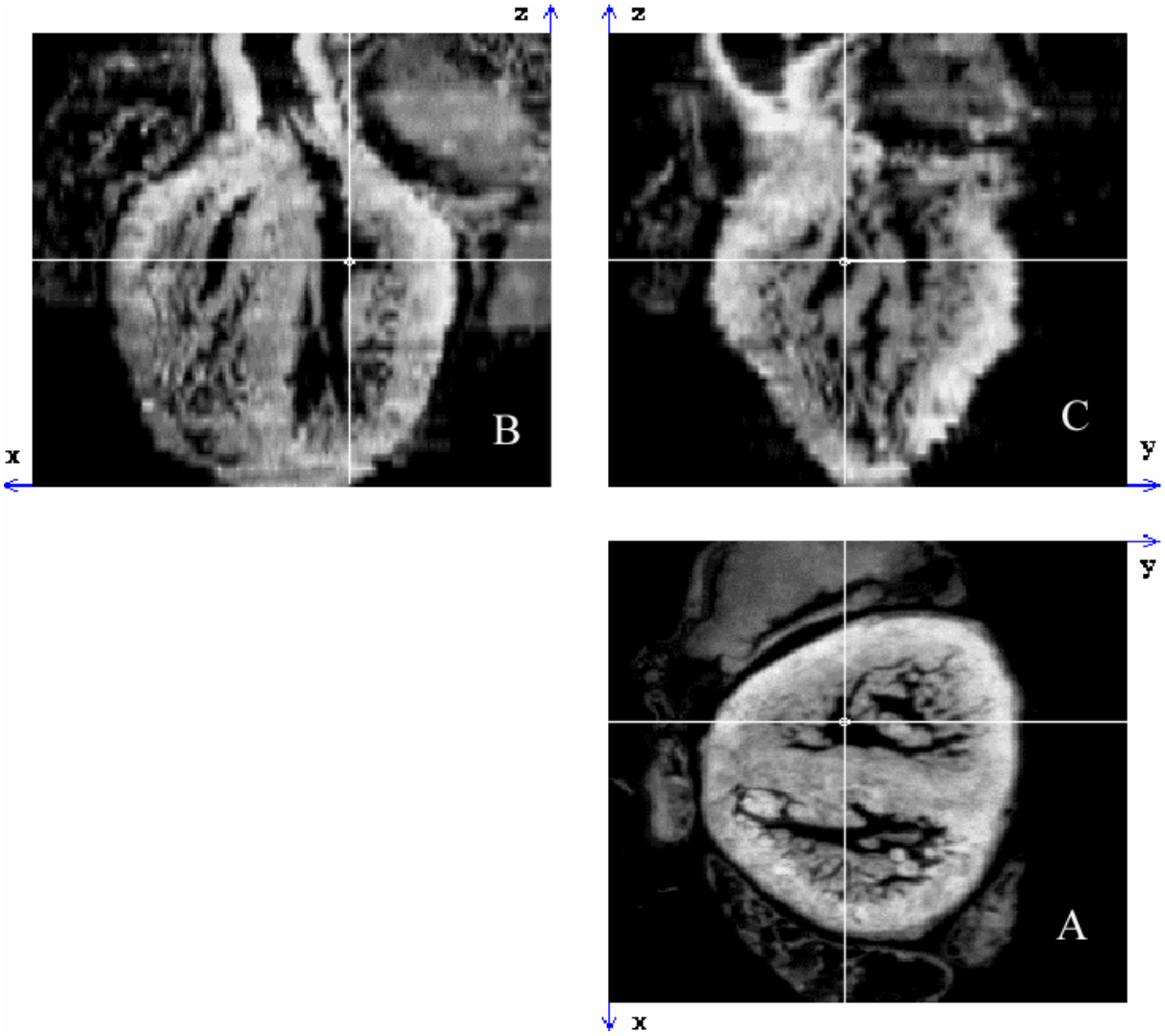

Figure 9

Figure 10

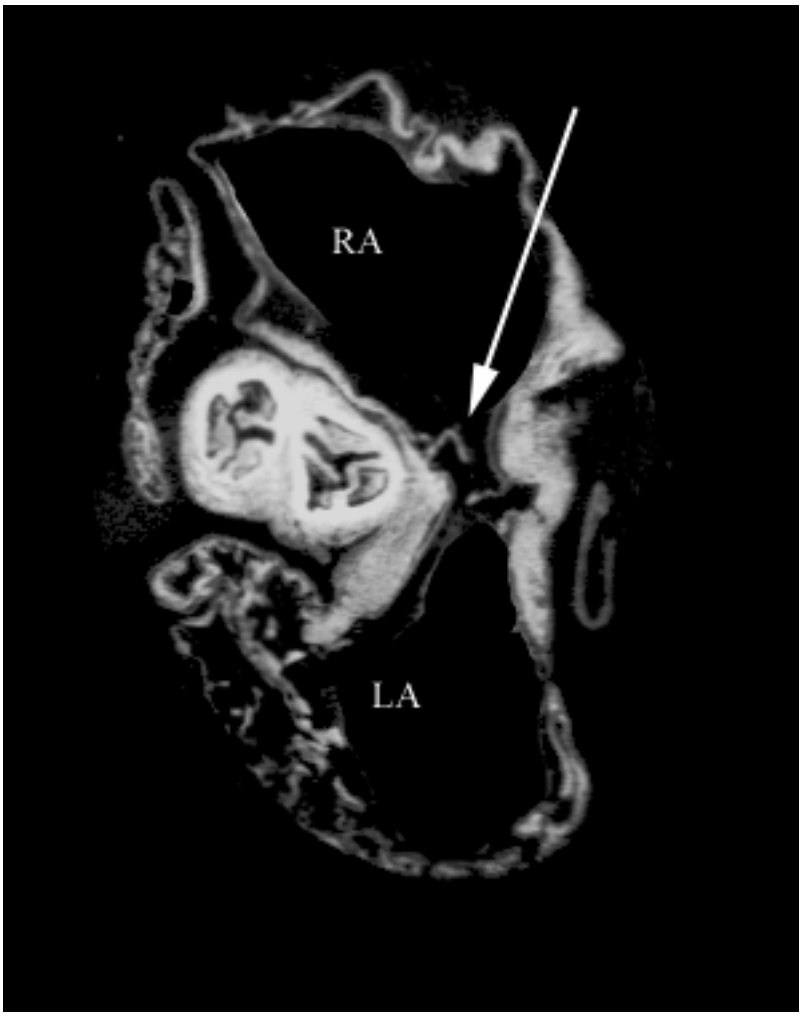

Figure 11

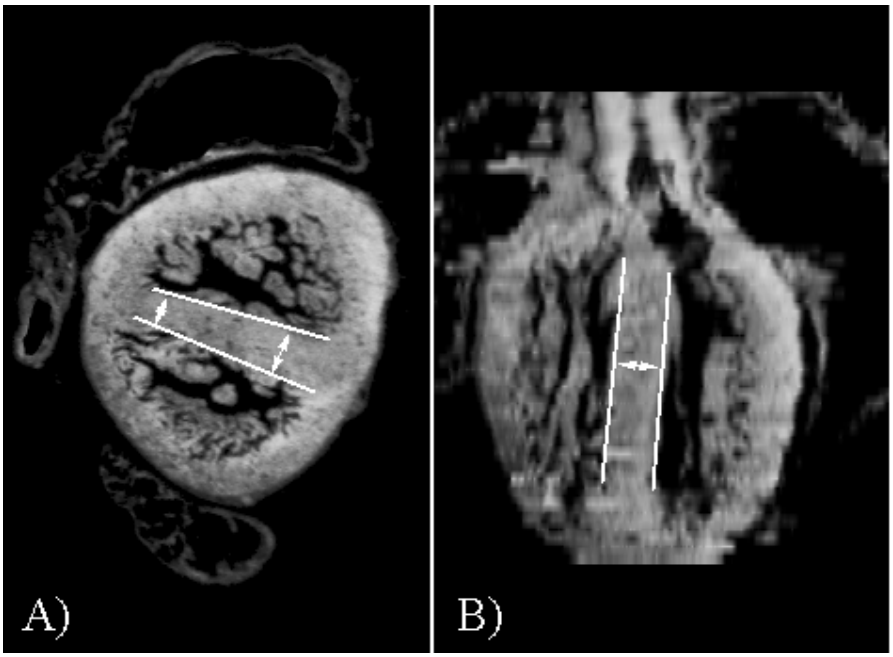

Figure 12